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METHODS IN CLINICAL PHARMACOLOGY

Development of an *in vivo* target-engagement biomarker for TRPA1 antagonists in humans

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AIM

To develop a non-invasive, safe and reproducible target-engagement biomarker for future TRPA1 antagonists in healthy volunteers.

METHODS

Dose finding (n = 11): 3%, 10%, and 30% cinnamaldehyde (CA) and placebo (= vehicle) was topically applied on the right forearm. One-way ANOVA with post-hoc Bonferroni was used to compare between doses. *Reproducibility*: 10% CA doses were topically applied during one visit on both arms (n = 10) or during two visits (n = 23) separated by a washout period of 7 days. CA-induced dermal blood flow (DBF) was assessed by laser Doppler imaging (LDI) at baseline and at 10, 20, 30, 40 and 50 min post-CA. Paired t-test was used to compare between arms or visits. Concordance correlation coefficient (CCC) was calculated to assess reproducibility. Data are expressed as percent change from baseline (mean ± 95% CI).

RESULTS

All three doses increased DBF compared to vehicle at all time-points, with the maximum response at 10–20 min post-CA. Dose response was found when comparing $AUC_{0–50min}$ of 30% CA (51 364 ± 8475%*min) with 10% CA (32 239 ± 8034%*min, P = 0.03) and 3% CA (30 226 ± 11 958%*min, P = 0.015). 10% CA was chosen as an effective and safe dose. DBF response to 10% CA was found to be reproducible between arms ($AUC_{0–50min}$, CCC = 0.91) and visits ($AUC_{0–50min}$, CCC = 0.83). Based on sample size calculations, this model allows a change in CA-induced DBF of 30–50% to be detected between two independent groups of maximum 10–15 subjects with 80% power.

CONCLUSIONS

Evaluation of CA-induced changes in DBF offers a safe, non-invasive and reproducible target-engagement biomarker *in vivo* in humans to evaluate TRPA1 antagonists.

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WHAT IS ALREADY KNOWN ABOUT THIS SUBJECT

- Cinnamaldehyde, the main component of cinnamon, activates the TRPA1 receptor and induces vasodilatation when applied on the human skin.
- TRPA1, a non-selective cation channel, is expressed in small diameter nociceptors and involved in persistent to chronic painful states such as inflammation, neuropathic pain and migraine.
- TRPA1 is an emerging target for treating these and other neurogenic inflammatory conditions.

WHAT THIS STUDY ADDS

- Cinnamaldehyde 10% topical solution is tolerable and safe to use in healthy volunteers.
- Cinnamaldehyde 10% applied on the human skin induces a robust increase in dermal blood flow which can be measured with laser Doppler imaging and is reproducible over time and between arms.
- The cinnamaldehyde model can be used in future early clinical development studies with TRPA1 antagonists as a target engagement biomarker to guide dose selections for efficacy studies.

Tables of Links

TARGETS	
Voltage-gated ion channels [2]	**Enzymes** [4]
TRPA1	Neuronal NOS
TRPV1	Prostaglandines
GPCRs [3]	
CGRP receptor	

LIGANDS
Cinnamaldehyde
Capsaicin
CGRP
Substance P
Nitric oxide

These Tables list key protein targets and ligands in this article that are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY [1], and are permanently archived in the Concise Guide to PHARMACOLOGY 2015/16 [2–4].

Introduction

Cinnamaldehyde (CA), the main component of Cinnamomi Cortex, is commonly used for flavouring and fragrance in foods, cosmetics and as a herbal medicinal product to treat infections, arthritis and cardiovascular diseases [5–7]. Cinnamaldehyde activates the transient receptor potential ankyrin type 1 receptor (TRPA1), a nonselective cation channel that belongs to the transient receptor potential (TRP) superfamily. Transient receptor potential channels (TRP channels) are a group of ion channels that are ubiquitously expressed in many cell types and tissues, both neuronal and non-neuronal. TRPA1 is often co-expressed with the transient receptor potential vanilloid type 1 receptor (TRPV1) in small to medium-diameter nociceptors of the dorsal root, trigeminal, jugular and nodose ganglia. As a result, they co-localize with peptidergic markers such as calcitonin gene-related peptide (CGRP), prostaglandins (PGs) and substance P (SP) [8–11]. Consequently, activation of TRPA1 by CA results in the release of these inflammatory mediators and neuropeptides which, in turn, impact surrounding tissues including: mast cells, immune cells and vascular smooth muscle cells. The resulting response encompasses redness and warmth due to vasodilatation and hypersensitivity due to excitation of primary sensory neurons; a response which is commonly referred to as 'neurogenic inflammation' [12, 13]. This makes TRPA1 a promising target for anti-inflammatory and analgesic drugs, for example for the treatment of migraine [14, 15]. Interestingly, TRPA1 is also expressed in non-neural cells of the human skin and involved in keratinocyte differentiation and dermal inflammatory responses [16, 17].

Preclinically, it has been shown that CA injection causes an increase in blood flow in the skin of anesthetized wild-type (WT) mice but not in TRPA1 knockout (KO) mice [18]. Likewise, the topical application of CA caused a TRPA1-dependent acute inflammatory response characterized by oedema formation and leukocyte infiltration [19]. Aubdool *et al.* showed that CGRP plays a key role in this murine TRPA1 activation, but also nitric oxide (NO) derived from neuronal nitric oxide synthase (nNOS) is involved [20]. The first human data were reported by Namer *et al.*, who demonstrated that the topical application of 10% CA on the forearm of healthy volunteers elicited a burning pain sensation as well as heat and mechanical hyperalgesia [21, 22]. Additionally, an exploratory study in human volunteers showed that the topical application of 1% CA to the forearm caused intense local cutaneous erythema and vasodilatation accompanied by a selective and dose-dependent release of PGD2 at the site of application [23].

Taken together, these findings indicate the potential of TRPA1 as a target for persistent to chronic painful states such as inflammation, neuropathic pain and migraine [18]. However, the transition between preclinical and clinical development is likely the biggest hurdle in the development of novel analgesics. The use of target engagement biomarkers

can potentially reduce this hurdle by measuring the interaction between the investigational medicinal product and the claimed target in the human body [24]. For example, the capsaicin model, developed for the activation of TRPV1 and release of CGRP, has been used extensively to guide dosing decisions in the early clinical development of TRPV1 antagonists and therapeutics interfering with CGRP or CGRP receptors [25].

The aim of this study was to develop a non-invasive, safe and reproducible target-engagement biomarker for future TRPA1 antagonists. To that end, cinnamaldehyde (CA)-induced dermal blood flow was evaluated using laser Doppler imaging (LDI) in healthy volunteers.

Methods

Subjects

Approval was obtained from the Ethics Committee of the University Hospitals Gasthuisberg, Leuven, Belgium (S54787/ML8725). All study procedures were carried out in accordance with the 2013 Declaration of Helsinki. Subjects were recruited via an approved database available at the Centre for Clinical Pharmacology. Written informed consent was obtained from all subjects prior to screening. All subjects were healthy (based on medical history), Caucasian, non-smoking volunteers between the age of 18 and 45 years. They could not have any skin disorders, excessive forearm hair growth, or allergies to any of the investigational products used, and were not taking any medication throughout the duration of the study.

Subjects were told to refrain from alcohol and caffeine consumption, to avoid use of topical treatments or lotions on the forearm 12 h prior to each study visit and to fast 3 h prior to each study visit.

Study design

Dose–response testing included one study visit during which three different doses of CA (3%, 10% and 30%) and vehicle were applied on the right forearm. Doses were based on the preclinical experiments of Aubdool *et al*. [20] and the clinical study of Namer *et al*. [21]. Inter-arm reproducibility testing included one study visit during which one dose of 10% CA and one dose of vehicle was applied on the left and right arms. Inter-period reproducibility testing included two study visits; during each visit one dose of 10% CA and one dose of vehicle was applied on the right forearm. Visits were separated by a washout period of at least 7 days. Visit were randomly planned in the morning or afternoon.

During each study visit, subjects rested in a semi-recumbent position on a comfortable bed in a quiet, temperature-controlled room (23°C ± 1°C) where all measurements were performed. Subjects underwent 30 min of acclimatization during which study restrictions were checked and blood pressure and heart rate measurements (Omron®, Model M6, Digital automatic blood pressure monitor, IntelliSense™, Milton Keynes, UK) were performed.

After acclimatization, O-shaped rubber rings (10 mm diameter, Quad Ring BS011 NBR 70 Shore A; Polymax Ltd, Bordon, UK) were placed on the volar surface of the forearm at equidistant sites and away from visible veins (four rings on the right arm for dose-finding, two rings on both the right and left arms for inter-arm reproducibility and two rings on the right arm for inter-period reproducibility). The rubber O-rings served as reservoirs for the topical application of 20 µl of CA or vehicle; the O-rings were not attached to the skin to avoid adverse local skin reactions. The rings were always placed within the same 4 × 4 cm zones on the forearm with 1 cm between zones and the first zone starting 5 cm distal from the elbow. The forearms of the subjects were placed in U-shaped cushions to prevent movement. DBF was measured at baseline (i.e. prior to CA/vehicle application) and at 10, 20, 30, 40 and 50 min after CA/vehicle application, using laser Doppler imaging (LDI) (PIMII, Perimed®). The pain intensity induced by CA application was assessed using the numerical rating scale −11 (NRS-11) before each LDI scan. Subjects were asked to score their pain orally on a numeric scale from 0 to 10, where 0 means 'no pain at all' and 10 means 'the worst pain you can imagine'.

Cinnamaldehyde

CA 99% was obtained from Sigma-Aldrich N.V. (Bornem, Belgium) and dissolved in a 3:3:1 mixture of ethanol 100%, Tween −20 and distilled water to obtain 3%, 10% and 30% CA concentrations. The vehicle solution corresponded to the same 3:3:1 mixture of ethanol 100%, Tween −20, and distilled water without CA.

Statistics

For dose-finding, the mean DBF response within the area of the rubber O-ring of the right arm was measured at each time-point for 3%, 10% and 30% CA and vehicle. The DBF response was expressed as mean percent change from baseline ± 95% confidence interval (95% CI); the corresponding area under the curve up to 50 min after CA application ($AUC_{0–50min}$) was calculated as a summary measurement. One-way analysis of variance (ANOVA) with post-hoc Bonferroni correction was used to test for significant difference between doses. P-value < 0.05 was considered as significant.

For reproducibility, the mean DBF response within the area of the rubber O-ring at each time-point for both arms and periods was measured for 10% CA and vehicle. DBF response was expressed as mean percent change from baseline ± 95% CI and the corresponding $AUC_{0–50min}$ was calculated as a summary measurement. For each subject, the DBF response was compared between the left and right arm for inter-arm reproducibility and between visit 1 and visit 2 for inter-period reproducibility using paired t-test for normally distributed data and related-samples Wilcoxon signed rank test for non-normally distributed data. The Shapiro–Wilk Test was used to assess the normality of the data distribution. P-value < 0.05 was considered as significant. Significant outliers were excluded using Cook's distance before proceeding with the reproducibility tests.

Test–retest reproducibility was further assessed using the mean difference, the concordance correlation coefficient (CCC), Bland–Altman graphs and the Bradley-Blackwood tests. The CCC assesses both accuracy and precision; a value closer to 1 indicates a more reproducible response. Bland–Altman plots were created to assess the agreement of

the individual mean DBF responses between arms or visits (pairwise means) *vs.* the difference in individual DBF responses between arms or visits (pairwise difference). The corresponding Bradley-Blackwood test gives an estimate whether the regression coefficients in the regression of the pairwise difference *vs.* the pairwise means are significantly different from zero [26]. Sample size calculations (SSC) for independent sample *t*-tests with continuous response measures were performed using PS: Power and Sample Size Calculator® software. Sample sizes required to detect a predetermined difference of 10, 20, 30 and 50% in DBF response given a significance level of 5% and a power of 80% were calculated. The active/vehicle ratio was assumed to be 1.

Drug/molecular target nomenclature is based on the BJP's Concise Guide to PHARMACOLOGY 2015/16 [27].

Results

Safety. Topical applications of 3% and 10% of CA on the forearm were well tolerated by all subjects. With the 30% CA dose, a mild and transient dermatitis reaction was reported in two subjects. In most subjects, 10 and 30% CA provoked a very mild itching sensation about 10 min post-application. This itching sensation disappeared within 20–30 min. Subjects gave NRS-11 scores ranging from 0–5 after 10 and 20 min and all scores were 0 at the subsequent time-points for all CA and vehicle doses. No significant differences were found between NRS scores for CA 3% (1.2 ± 1.0; 0.18 ± 0.6), 10% (1.2 ± 1.0; 0.2 ± 0.6), 30% (1.2 ± 1.0; 0.3 ± 0.7) or vehicle (0.6 ± 1.1; 0 ± 0) at 10 or 20 min, respectively (P = 0.893; P = 0.591; Kruskal-Wallis test with Dunn's correction for multiple comparisons). All three doses of CA elicited a transient local redness at the site of application, which disappeared within hours after completion of the measurements. Based on these data, 3% and 10% CA were considered safe to use in humans.

Efficacy

Dose-finding. Dose-finding experiments were performed in a total of 11 healthy Caucasian volunteers, of which nine were male. Mean ± SD (range) for age, body mass index (BMI), systolic blood pressure, diastolic blood pressure and heart rate were: 26 ± 3 (22–33) years, 22 ± 3 (17–26) kg m^{-2}, 118 ± 9 (104–132) mmHg, 71 ± 6 (60–73) mmHg and 67 ± 7 (57–81) bpm, respectively.

An increase in DBF (expressed as % change from baseline ± 95% CI) was observed for all three doses of CA, starting from 10 min post-CA application and gradually declining thereafter (Figure 1A). The increase in DBF was different from vehicle for all three doses at all time-points ($P < 0.001$, one-way ANOVA with Bonferroni correction for multiple testing). The maximum DBF was observed at 10 min after 30% CA (1292 ± 229%); at 20 min after 10% CA (1042 ± 238%) and 3% CA (938 ± 354%) application. For all doses, the DBF response was slowly returning to baseline at 50 min post-dose. However, no LDI measurements were done after this time-point. Based on visual inspection of the forearms, the increased DBF was back to baseline after approximately 60 min. The most robust DBF response was induced by the 30% CA dose. The DBF response induced by 30% CA was significantly different from that of 3% CA at all time-points: 1292 ± 229 *vs.* 864 ± 375% at 10 min (P = 0.01), 1265 ± 228 *vs.* 938 ± 354% at 20 min (P = 0.04), 1197 ± 189 *vs.* 867 ± 332% at 30 min (P = 0.02), 1046 ± 195 *vs.* 724 ± 323% at 40 min (P = 0.01) and 708 ± 163 *vs.* 518 ± 279% at 50 min (P = 0.04, one-way ANOVA with post-hoc Bonferroni). No significant difference was observed between the DBF response induced by 30% CA and 10% CA or between 10% CA and 3% CA at any of the time-points.

When the DBF response was expressed as area under the curve from 0 to 50 min ($AUC_{0–50min}$, %change from baseline*min), the DBF induced by 30% CA (51 364 ± 8475%*min) was higher than that of 10% CA (32 239 ± 8034%*min, P = 0.02) and 3% CA (30 226 ± 11 958%*min, $P < 0.008$) (Figure 1B). $AUC_{0–50min}$ was higher compared to vehicle (616 ± 205%*min) for all doses ($P < 0.001$). There was no significant difference between the DBF (AUC) response between the 10% and 3% CA ($P > 0.05$, one-way ANOVA with post-hoc Bonferroni). The same results were found when analyzing the DBF in the flare region (= outside the rubber O-ring) (Suppl. Figure S2).

Because 30% CA caused local dermatitis in two subjects and 3% CA showed larger inter-subject variability, the 10% CA dose was chosen as a safe and efficacious dose to further develop the model as a biomarker and to investigate the reproducibility.

Intra-subject inter-period and inter-arm reproducibility. **The inter-arm reproducibility** (Figure 2B) was completed in 10 healthy Caucasian volunteers, of which six were males. Mean ± SD (range) for age, BMI, systolic blood pressure, diastolic blood pressure and heart rate were 23 ± 2 (19–25) years, 21 ± 3 (19–25) kg m^{-2}, 119 ± 13 (99–138) mmHg, 73 ± 7 (63–82) mmHg and 76 ± 9 (63–93) bpm, respectively.

There was no difference in DBF response to 10% CA between the right and left arms at 10 min (1087 ± 372 *vs.* 1009 ± 349%, P = 0.60), 20 min (1181 ± 405 *vs.* 1236 ± 363%, P = 0.80), 30 min (1084 ± 401 *vs.* 1135 ± 365%, P = 0.91), 40 min (921 ± 418 *vs.* 992 ± 414%, P = 0.44), and 50 min (642 ± 356 *vs.* 686 ± 346%, P = 0.26) (paired *t*-test). The maximum DBF response was observed at 20 min in both the right (1181 ± 405%) and left arms (1236 ± 363%) (Figure 2A). The CCC was >0.8 (= substantial–almost perfect) for all time-points except at 10 min post-CA (CCC = 0.74, moderate) (Table 1). Vehicle did not induce a DBF response at any of the time-points and was identical between the right and left arms ($P > 0.05$, paired *t*-test). $AUC_{0–50min}$ of the CA-induced DBF did not differ between the arms (38 127 ± 13 696 *vs.* 38 771 ± 11 946%*min, P = 0.80, paired *t*-test). Based on the CCC (0.91) and Bradley-Blackwood test (P = 0.30), $AUC_{0–50min}$ was found to be reproducible between arms.

Sample size calculations showed that the CA model is able to detect a change of 30–50% in DBF between two independent groups with a maximum of 10 subjects when comparing between arms (Table 2).

The inter-period reproducibility (Figure 2A) was completed in 25 healthy Caucasian volunteers, of which 13 were males. Mean ± SD (range) for age, BMI, systolic blood pressure, diastolic blood pressure and heart rate were 24 ± 5

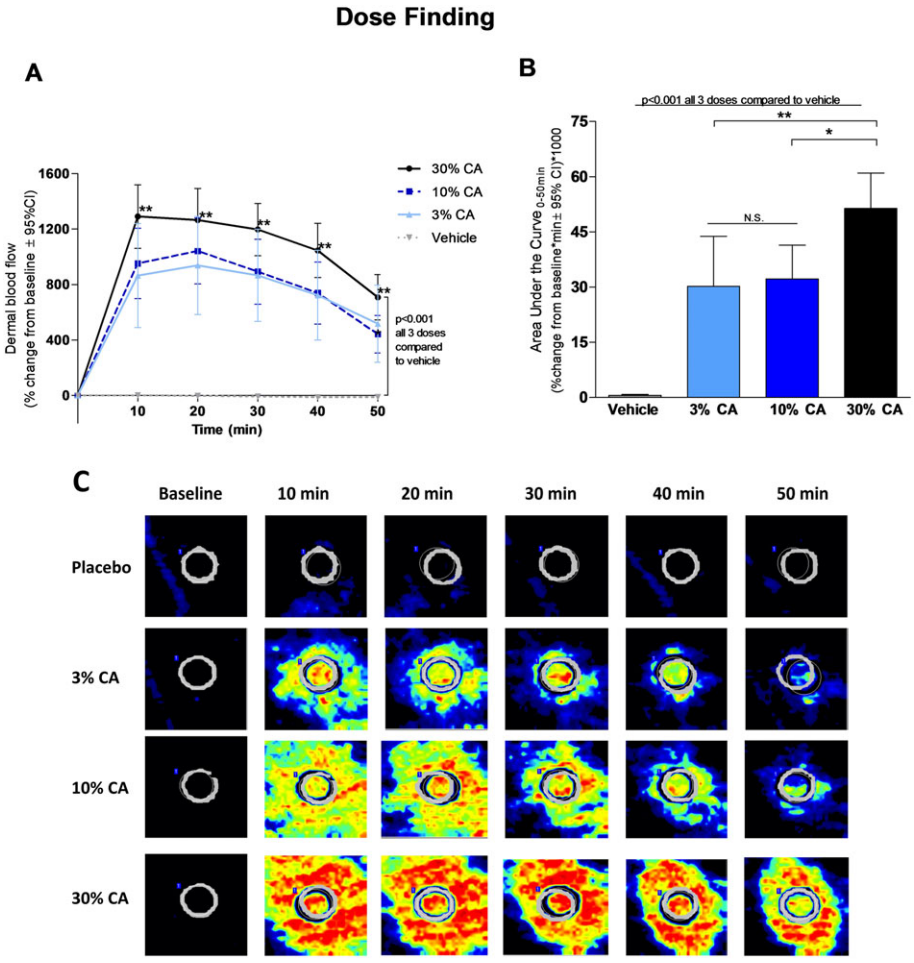

Figure 1

Dose finding of the DBF response after vehicle and 3%, 10% and 30% cinnamaldehyde (CA) application, expressed as % change from baseline over time (A), and expressed as area under the curve (AUC) from 0–50 min (B), in healthy volunteers (n = 11) and shown as LDI images in one healthy volunteer (C)(A) and (B): *$P < 0.05$ when comparing 10% to 30% CA; **$P < 0.05$ when comparing 3% to 30% CA; $P < 0.001$ when comparing 3%, 10%, 30% CA to vehicle (one-way ANOVA with post-hoc Bonferroni). N.S.: non-significance. Data expressed as mean ± 95% CI

(19–46) years, 22 ± 3 (17–25) kg m^{-2}, 117 ± 10 (99–138) mmHg, 72 ± 7 (60–82) mmHg, 72 ± 11 (50–93) bpm, respectively. As two outliers (one male, one female) were eliminated from further statistical tests based on Cook's distance test, 23 subjects were included in the final inter-period reproducibility analysis.

There was no difference in the CA-induced DBF response between visit 1 and visit 2 after 10 min (907 ± 189 *vs.* 966 ± 181%, P = 0.69), 20 min (1091 ± 194 *vs.* 1099 ± 245%, P = 0.10), 30 min (1000 ± 205 *vs.* 1048 ± 290%, P = 0.31), 40 min (808 ± 214 *vs.* 778 ± 226%, P = 0.22), and 50 min (480 ± 161 *vs.* 484 ± 181%, P = 0.59) (paired t-test). The maximum DBF response was observed at 20 min in both visit 1 (1091 ± 194%) and visit 2 (1099 ± 245%). The CCC varied from 0.65 to 0.8 (= moderate) at 20 and 50 min post-CA, CCC was below 0.65 (= poor) at 10, 30 and 40 min post-CA (Table 1). Vehicle did not induce a DBF response at any of the time-points and was identical between visit 1 and visit 2 ($P > 0.05$, paired t-test). $AUC_{0–50min}$ of the CA-induced DBF was not different between visit 1 and visit 2 (24 988 ± 4490 *vs.* 25 836 ± 5285%*min, P = 0.568, paired t-test). Based on the CCC (0.83) and Bradley-Blackwood test (P = 0.18), $AUC_{0–50min}$ was found to be reproducible between visits.

Sample size calculations showed that the CA model is able to detect a change of 30–50% in DBF between two independent groups with a maximum of 15 subjects when comparing between visits (Table 2).

Additional reproducibility analysis and sample size calculations are shown in Tables 1 and 2.

Discussion

The use of cinnamaldehyde-induced changes in dermal blood flow is presented as a safe, non-invasive and reproducible target-engagement biomarker to test future TRPA1 antagonists.

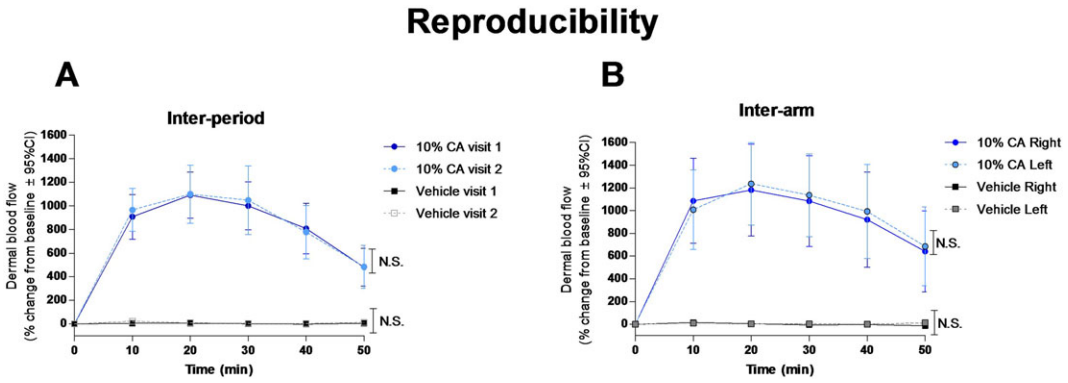

Figure 2

(A) Comparison of the DBF response after 10% CA between visit 1 and visit 2 and after vehicle between visit 1 and visit 2, i.e. inter-period reproducibility ($n = 23$). (B) Comparison of the DBF response after 10% CA between right and left arm and after vehicle between right and left arm, i.e. inter-arm reproducibility ($n = 10$). N.S.: non-significance ($P > 0.05$, student paired t-test to compare between arms and periods). Data expressed as mean ± 95% CI

Table 1

Test–retest reproducibility of dermal blood flow response induced by 10% CA based on CCC and BB test

	Inter-arm reproducibility ($n = 10$)		Inter-period reproducibility ($n = 23$)	
Time	**CCC**	**BB test**[a]	**CCC**	**BB test**[a]
10 min	0.74	0.7921	0.62	0.5965
20 min	0.84	0.5694	0.73	0.0886
30 min	0.95	0.3355	0.45	0.0664
40 min	0.99	0.8500	0.55	0.8112
50 min	0.99	0.5808	0.74	0.4362

Test–retest inter-period and inter-arm reproducibility for DBF response expressed as percentage change in baseline at 10, 20, 30, 40 and 50 min after CA application
CCC: concordance correlation coefficient
CCC > 0.9, almost perfect; CCC 0.8–0.9, substantial; CCC 0.65–0.8, moderate; CCC < 0.65, poor
[a]Bradley-Blackwood test: $P < 0.05$ indicates evidence of unequal means or unequal variances between period 1 and period 2 or between right arm and left arm

First, in the dose-finding part of the study, it was shown that all three doses of CA increased DBF compared to vehicle at all time-points, with the maximum response at 10–20 min post-CA application. A difference in dermal blood flow response was found when comparing $AUC_{0-50min}$ of 30% CA with 10% CA and 3% CA. At all time-points measured (10–50 min post-CA application), a difference was found between 30% CA and 3% CA. No difference was found between 10% and 3% CA which is likely due to the high inter-subject variability of the 3% dose. A transient dermatitis at the site of application was caused by 30% CA in two volunteers, whereas 3% and 10% CA caused limited to no irritation. NRS-11 pain scores were less than 5 in all subjects. Hence, 10% CA was the optimal and safest dose to continue with, as it produces a robust DBF response with low inter-individual variability as well as minimal skin irritation. These findings seem to be in line with the study of Namer *et al*. where 10% CA was also used; however, they expressed DBF as area of vasodilatation and not as perfusion units.

Second, in the reproducibility part of this study, the DBF response to 10% CA was found to be reproducible between arms as well as between visits. Reproducibility between arms was better compared to inter-period reproducibility. This slightly lower inter-period reproducibility may be attributed to the diurnal variation in forearm blood flow, which has been shown to be lower in the morning and increased by 58% during the day [28]. Several mechanisms may explain the circadian pattern of forearm blood flow, including increased α-sympathetic vasoconstrictor activity in the morning, which affects the vasculature tone. Skin temperature has also been shown to be associated with dermal blood flow, and skin temperatures are slightly lower in the mornings. As a result, there is a lower metabolic rate in the early hours and thus less demand for nutrients to be delivered via the blood

Table 2

Sample size calculations for dermal blood flow response induced by 10% CA

DBF response	Test–retest	Mean difference (95% CI)	CCC	SSC 10% shift	SSC 30% shift	SSC 50% shift	Shapiro–Wilk test[a]	BB test[b]
AUC_{0-50} (%*min)	Inter-arm (n = 10)	−1121 (−7490, 5249)	0.91	79	10	4	0.21	0.30
	Inter-period (n = 23)	−848 (−3881, 2185)	0.83	117	14	6	0.64	0.18
t_{30} (%)	Inter-arm (n = 10)	−51 (−177, 75)	0.95	40	5	3	0.0777	0.3355
	Inter-period (n = 23)	−62 (−313, 1878)	0.45	551	63	23	0.0002	0.0664

Test–retest inter-period and inter-arm reproducibility data for DBF response expressed as percentage change in baseline at 30 min (t_{30}) and as AUC of percent change from baseline from 0 min to 50 min (AUC_{0-50})
CCC, concordance correlation coefficient
CCC > 0.9, almost perfect; CCC 0.8–0.9, substantial; CCC 0.65–0.8, moderate; CCC < 0.65, poor
SSC: sample size calculation to detect an X% shift between two independent groups with an 80% power, a 5% significance level, and an active/placebo ratio of 1
[a]Shapiro–Wilk test: $P > 0.05$ indicates normal distribution of data
[b]Bradley-Blackwood test: $P < 0.05$ indicates evidence of unequal means or unequal variances between visits or arms

flow. In our study, the LDI measurements for each subject were not necessarily made at the same time of the day in visit 1 and visit 2, which may have led to a slight difference in DBF response and increased variability. To minimize the influence of skin temperature, measurements were always performed at a standardized temperature (23°C ± 1°C) and after sufficient acclimatization.

Regarding the reproducibility calculations, CCC and Bradley Blackwood in combination with paired *t*-tests were chosen as preferred parameters, as recommended by Russell *et al*. [29]. The Bradley-Blackwood test is easy to implement and corresponds with the graphical methods of Altman and Bland [30]. CCC is a reliable measure of reproducibility that measures the agreement between two variables by assessing the degree to which pairs of observations fall on the 45° line through the origin. It is essentially a modified version of the Pearson coefficient of correlation, which measures how close the data points fall relative to the line of best fit. CCC is superior in the sense that it takes into account how far the line of best fit lies from the 45° line through the origin [31].

Based on these reproducibility calculations, we were able to calculate the sample size, which showed that the CA model is able to detect a change of 30–50% between two independent groups of 15 subjects with 80% power. However, it is important to keep in mind that every model is only a surrogate of reality and has its limitations. Because TRPA1 antagonists are not yet available for human use, we were not able to test the specificity of the response to CA for TRPA1 in humans. However, extensive preclinical experiments support this specificity and currently no evidence for species differences in CA sensitivity has been reported [12, 32, 33]. In line with other DBF models such as the capsaicin model, one can assume that several factors such as sex, age and body site can play a role in the response to CA. Our sample of healthy subjects consisted of males and females within a predefined age range. Interestingly, no significant difference was found between males and females (suppl. Figure S1), although, at 20 min, there seems to be a trend (unpaired *t*-test, $P = 0.12$) towards a higher response in women. However, this is probably due to a small, nonsignificant difference in baseline DBF (32 PU (females) *vs*. 45 PU (males); $P = 0.4$, unpaired *t*-test). Only the volar surface of the forearm was used for application of CA; as a consequence, our model only represents peripheral activation of TRPA1 which can most obviously be used to investigate TRPA1-related peripheral disorders. In the context of migraine, where trigeminal activation of TRPA1 is hypothesized to play an important role, it would also be interesting to evaluate the application of CA on the skin of the forehead in order to activate trigeminal TRPA1 channels. If central rather than peripheral activation of TRPA1 channels is involved in migraine, the added value of CA-induced changes in DBF as a biomarker for target engagement could be questioned. However, also in that case, CA-induced changes in DBF as a biomarker could still provide very useful information to guide dose selection for efficacy studies.

Unlike other non-invasive DBF models such as the capsaicin model, no non-responders (i.e. DBF increase of <100% compared to baseline) were reported with the 10% CA model. This gives the CA model an extra advantage as a target-engagement model in future clinical research. Moreover, there seems to be no desensitization of the CA response after repeated administration, which is in line with preclinical findings in mice [19]. Another unresolved question is whether the DBF response observed is due to activation of neuronal and/or vascular TRPA1. Based on preclinical findings of Aubdool *et al*., it seems that neuronal TRPA1 is activated, which in turn causes the release of several neuropeptides including CGRP, Substance P and prostaglandins, and are most likely to play an important role in the neurogenic inflammation reaction [12, 32–34]. Therefore, future research questions still to be looked into are: (1) which secondary messengers are involved in the DBF response after

CA application *in vivo* in humans, and (2) what is the influence of TRPV1 co-activation. Additional topics for further investigation are potential differences in the response to CA between different populations (e.g. migraine patients) and possible within-subject differences in response to CA depending on the area of application. As there is growing evidence that targeting TRPA1-mediated neurogenic inflammation of the trigeminal system might be beneficial in migraine [14, 15], this non-invasive model will be useful for testing novel anti-migraine and analgesic drugs linked with TRPA1 and CGRP.

Taken together, we have developed a target-engagement biomarker to test TRPA1 antagonists *in vivo* in humans that is non-invasive, reproducible and safe. The model provides an objective pharmacodynamic endpoint, which is easy to incorporate in phase I clinical trials. The use of this model in exploratory clinical trials with TRPA1 antagonists and/or related mediators is an extra asset to facilitate dose selection and go/no go decisions in early clinical drug development.

Competing Interests

All authors have completed the Unified Competing Interest Form at www.icmje.org/coi_disclosure.pdf (available on request from the corresponding author) and declare: no support from any organization for the submitted work, JdH was Principal Investigator for clinical studies commissioned by Amgen Inc., Eli Lilly and Company and Merck Sharp & Dohme in the previous 3 years; no other relationships or activities that could appear to have influenced the submitted work.

Contributions

L.B., L.A and L.C performed the study assessments. J.d.H. was the Principal Investigator of the study. All authors contributed in writing and supervising the manuscript.

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

http://onlinelibrary.wiley.com/doi/10.1111/bcp.13143/suppinfo

Figure S1 The CA-induced DBF response in healthy males ($n = 11$) *vs*. females ($n = 12$), expressed as % change from baseline over time. N.S.: non-significance (unpaired *t*-test). Data expressed as mean ± 95% CI

Figure S2 Dose-finding of the DBF response in the flare region (= outside the rubber O-ring) after vehicle and 3%, 10% and 30% cinnamaldehyde (CA) application, expressed as % change from baseline over time (A), and expressed as area under the curve (AUC) from 0–50 min (B), in healthy volunteers ($n = 11$) and shown as LDI images in one healthy volunteer (C). * $P < 0.05$ when comparing 10% to 30% CA; ** $P < 0.05$ when comparing 3% to 30% CA; $P < 0.001$ when comparing 3%, 10%, 30% CA to vehicle (one-way ANOVA with post-hoc Bonferroni). N.S.: non-significance. Data expressed as mean ± 95% CI